Remarks

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

Claims 3 and 5 have been amended. Descriptive support for the amendments to claims 3 and 5 is provided in original claim 5 as well as page 7, lines 5-15, page 8, lines 1-8, and the Examples (*see* Example 8 re: adenocarcinoma). Therefore, no new matter has been introduced by these amendments.

Claims 3-9, 12, and 13 remain pending, with claims 3-5, 7-9, 12, and 13 under examination and claim 6 standing withdrawn.

The rejection of claims 3, 4, and 7 under 35 U.S.C. § 102(a) as anticipated by Murdaca et al., *AIDS* 16(2):304-5 (2002)("Murdaca") is respectfully traversed.

At page 3 of the office action, the U.S. Patent and Trademark Office ("PTO") asserts that Kaposi's sarcoma ("KS") is an epithelial tumor. While applicants disagree with this interpretation by the PTO because Kaposi's sarcoma is a mesenchymal tumor, to clarify the claim language applicants have amended claim 3 to recite "carcinoma" rather than "epithelial tumor." KS is not a carcinoma, nor is it a tumor of the nervous system, a form of leukemia, or a fibro- or osteo-sarcoma. Therefore, the claims do not read on the treatment of KS as taught in Murdaca. For this reason, the rejection of claims 3, 4, and 7 as anticipated by Murdaca is improper and should be withdrawn.

The rejection of claims 8 and 9 under 35 U.S.C. § 103(a) for obviousness over Murdaca in view of U.S. Patent No. 6,235,733 to Bahal et al., ("Bahal") is respectfully traversed.

The teachings and deficiencies of Murdaca are noted above. As noted above, Murdaca discloses only the treatment of a patient suffering from AIDS and the related KS tumor. There is no mention of any other types of tumor. Bahal is cited at page 10 of the office action for teaching oral liquid formulations of efavirenz; however, the PTO has failed to demonstrate how Bahal overcomes the above-noted deficiencies of Murdaca. Therefore, the obviousness rejection of claims 8 and 9, which ultimately depend from claim 3, is improper and should be withdrawn.

The rejection of claims 3, 4, and 7 under 35 U.S.C. § 103(a) for obviousness over Grimaudo *et al.*, *Eur. J. Cancer* 34:1756-1763 (1998) ("Grimaudo") is respectfully traversed.

Grimaudo teaches the use of 1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo[3,4-a]benzimidazole ("TBZ") to induce apoptosis in treated HL60 leukemia cells.

At page 7 of the office action, the PTO asserts that one of skill in the art would have been motivated to "utilize the specific non-nucleoside reverse transcriptase inhibitors, because Grimaudo et al. render the administration of non-nucleoside reverse transcriptase inhibitor to treat leukemia obvious. Accordingly, one would have had an expectation of similar success in treating leukemia with ...efavirenz, as instantly claimed." Applicants respectfully disagree for several reasons.

Firstly, the PTO improperly asserts that motivation for treating leukemia in accordance with Grimaudo is all that is required to reach the conclusion of obviousness and, based on this prior determination of obviousness, the expectation of success would have existed. Whether the prior art provides an expectation of success must be assessed *before* reaching a conclusion of obviousness. *See In re Vaeck*, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991). Consequently, applicants respectfully submit that the assertion of obviousness by the PTO is defective, and the *prima facie* obviousness rejection must be withdrawn.

Secondly, the results presented in Grimaudo are clearly limited to TBZ. Indeed, it is never even suggested in Grimaudo that other non-nucleoside reverse transcriptase inhibitors ("NNRTI") outside of thiazolo benzimidazoles might have the same anti-cancer effect. Quite the contrary, Grimaudo indicates that "synthetic efforts ...in progress to block [TBZ] metabolism with retention of activity" were underway due to the rapid inactivation of TBZ as observed by Grimaudo. This suggests that the authors of Grimaudo did not contemplate looking beyond TBZ derivatives, *i.e.*, other thiazolo benzimidazoles. Thus, the PTO has not and cannot cite any statement in Grimaudo to support its position.

Thirdly, one of ordinary skill in the art would not have generalized that NNRTI would have been useful for treating leukemia based on the teachings of Grimaudo. One reason for this is the fact that TBZ has a completely different structure from the compounds recited in claim 3. Neither nevirapine, efavirenz, delaviridine, nor 5,11-dihydro-*6H*-dipyrido-(3,2-b:2',3'-e)(1,4)-diazepine compounds are thiazolo benzimidazoles like TBZ. Another reason is that the compounds recited in claim 3 are known to bind the hydrophobic pocket on RT subunit p66, whereas no such information is provided for TBZ in Grimaudo. For this reason, one of ordinary skill in the art would not have expected that the structurally distinct TBZ would have the same properties as the compounds recited in claim 3. Moreover, the cytotoxic activity of TBZ on HL60 tumor cells, as measured by Grimaudo, was obtained at concentrations much higher than

those reported for antiviral activity. Indeed, it is indicated in the first paragraph of the discussion of Grimaudo on page 1760 that TBZ inhibits HIV-1-induced cell killing and viral replication in several human cell lines, as well as fresh human peripheral blood lymphocytes and macrophages at a concentration of about 1 μM. In contrast, as shown in Figure 2 of Grimaudo, the concentration of TBZ necessary to obtain at least 50% cytotoxicity is at least 75 μM. This suggests that the two effects rely on distinct mechanisms, *i.e.*, that the anti-tumor effect of TBZ on HL60 tumor cells is *not* related to its RT inhibitory activity. A further reason is that the results of Grimaudo indicate that TBZ acts on HL60 tumor cells by inducing apoptosis of these cells. Since RT inhibitory activity is *not* related to induction of apoptosis, Grimaudo clearly does not suggest that another RT inhibitor other than TBZ might have anti-tumor efficiency. Quite the contrary, the results of Grimaudo would have suggested to one of ordinary skill that TBZ, given its distinct structure and function, operated through a different mechanism or cellular pathway to achieve its results.

For all these reasons, applicants submit that a person of ordinary skill in the art would not have considered the subject matter of claims 3, 4, and 7 obvious over Grimaudo. Therefore, this rejection should be withdrawn.

The rejection of claims 3, 4, 5, 12, and 13 under 35 U.S.C. § 103(a) for obviousness over Ghori *et al.*, *Colorectal Disease* 2(2):106-112 (2000) ("Ghori") is respectfully traversed.

Ghori relates to the study of the action of nucleoside analogues (*i.e.*, not NNRTIs) on telomerase activity. Telomerase is an enzyme which is responsible for the prevention of the degradation of chromosomal telomeres. Although both telomerase and reverse transcriptase are capable of reverse transcribing DNA, they are completely different enzymes. In particular, HIV-1 RT is made of two subunits of 66 (p66) and 51 (p51) kDa, respectively, wherein the p66 subunit contains the DNA polymerase and RNase H domains, while the p51 subunit contains the DNA synthetic activity. *See* Tarrago-Litvak et al., "The Reverse Transcriptase of HIV-1: From Enzymology to Therapeutic Intervention," *FASEB J.* 8:497-503 (1994) (copy attached as Exhibit 1) at abstract. In contrast, telomerase is made of a RNA subunit and a catalytic RT subunit (designated TERT). *See* Nugent et al., "The Telomerase Reverse Transcriptase: Components and Regulation," 12:1073-1085 (1998) ("Nugent") (copy attached as Exhibit 2). Nugent clearly distinguishes telomerase from other RTs as follows:

- At page 1076, left column, first full paragraph, the authors assert: "The dependence of telomerase polymerase activity upon RNA formally defined the enzyme as a *specialized type* of reverse transcriptase (RT)" (emphasis added);
- At page 1077, left column, first paragraph, the authors assert: "However, despite the overall similarities with RNA-dependent polymerases, including the three aspartate residues required for enzyme catalysis, telomerases from disparate organisms are more related to one another than to other polymerases and thus *appear to form a distinct subgroup*" (emphasis added; citations omitted); and
- At page 1077, left column, second paragraph, the authors assert: "These variations in the features of the telomerase RT motifs may reflect the obvious mechanistic differences between this enzyme and conventional RTs."

Thus, the prior art clearly considers telomerases as a distinct and specialized class of reverse transcriptase. As such, the properties of this distinct class, and the effects of drugs on members of this distinct class, cannot necessarily be generalized to the larger class of RT.

Ghori discloses the results of treating the tumor cell line HT29, a human colon adenocarcinoma cell line, with various nucleoside analoges. A slower proliferation rate of the cells is observed. From this data, Ghori speculates that nucleoside analogues (but not NNRTIs) may be useful as agents for the treatment of colorectal cancers (*see* Ghori at p. 111). Even assuming this to be true, which applicants do not admit, then Ghori cannot be said to support treating even colorectal cancers with a different class of agents (the NNRTIs recited in claim 3) that are structurally distinct.

For these reasons, applicants submit that a person of ordinary skill in the art would not have considered the subject matter of claims 3, 4, 5, 12, and 13 obvious over Ghori. Therefore, this rejection should be withdrawn.

In view of the foregoing, it is submitted that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

Date: May 27, 2010

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Exhibit 1: Tarrago-Litvak et al., "The Reverse Transcriptase of HIV-1: From Enzymology to Therapeutic Intervention," *FASEB J.* 8:497-503 (1994)

The reverse transcriptase of HIV-1: from enzymology to therapeutic intervention

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ABSTRACT **The human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS. Replication of this virus requires the activity of a retrovirus encoded RNA-dependent DNA polymerase, or reverse transcriptase (RT). HIV-1 RT is required for the synthesis of the double-stranded proviral DNA from the single-stranded retroviral RNA genome. HIV-1 RT has two subunits of 66 kDa and 51 kDa. The 66-kDa subunit contains the DNA polymerase and RNase H domains whereas the 51-kDa subunit, obtained by proteolytic maturation of the former subunit, has only the DNA synthetic activity. Two recently reported crystal structures of HIV-1 RT have revealed the very asymmetric structure of this molecule. In addition to providing information concerning the mechanism of nucleic acid polymerization, biochemical and biophysical studies of this enzyme are providing key insights for the design of selective antiviral agents. The multiple activities displayed by reverse transcriptase in the replication of the retroviral genome ensure that this enzyme will remain at the forefront of antiviral strategies in the fight against AIDS and other retrovirus-related pathologies.—Tarrago-Litvak, L., Andréola, M.-L., Nevinsky, G. A., Sarih-Cottin, L., and Litvak, S. The reverse transcriptase of the human immunodeficiency virus type 1: from enzymology to therapeutic intervention. *FASEB J.* 8: 497–503; 1994.**

Key Words: structure-functional studies • 3-D structure • inhibitors

TEN YEARS AGO, ANNOUNCEMENTS IN PARIS and in Bethesda, Maryland, declared that a retrovirus had been isolated that was the etiologic agent of the acquired immunodeficiency syndrome (AIDS).[2] That announcement was followed by a massive effort to study this retrovirus, known today as the human immunodeficiency virus type 1 (HIV-1). Nevertheless, 5 years later a review in the *Journal of Biological Chemistry*, written by Arthur Kornberg, stated, "In view of the general concern with controlling AIDS, it is remarkable that so little is known about reverse transcriptases, and how they synthesize duplex DNA and prepare it for integration into a host chromosome. Not a single paper on reverse transcriptase appeared in this Journal in 1986" (1). As if in response to this reprimand, an increasing number of articles describing both the mechanism of action of this enzyme and the potential therapeutic action of many specific inhibitors appeared in biochemical journals. An important contribution to this field of study was the determination last year of the three-dimensional structure of the two enzymatic domains of the enzyme (see below).

Reverse transcriptase is a DNA polymerase: it synthesizes DNA using a complementary nucleic acid strand as template. Because it uses RNA as the template and synthesizes a complementary DNA strand, it is classified as an RNA-dependent DNA polymerase. During the life cycle of the virus, this enzyme is involved in copying the retroviral RNA genome into a double-stranded proviral DNA molecule, which is then integrated into the nuclear DNA of the transformed cell. Twice in the last 20 years reverse transcriptase has played a key role in promoting a massive research effort toward understanding the enzymology of DNA replication. This enzyme was first discovered in 1970 in the laboratories of Temin and Baltimore (2, 3). Its existence seemed to contradict the so-called "general dogma of molecular biology" in which the flow of genetic information is unidirectional, going from DNA to RNA to protein. Moreover, the discovery of this unique retroviral polymerase may have precipitated the subsequent identification and characterization, in the early 1970s, of the eucaryotic DNA polymerases α, β and the mitochondrial polymerase γ, followed many years later by the isolation of DNA polymerases δ and ϵ, also involved in nuclear DNA replication (for a recent review on eucaryotic DNA polymerases, see ref 4). In part because the very efficient utilization of the synthetic RNA, poly rA, as template by the mitochondrial DNA polymerase γ, this enzyme was initially mistaken for "cellular" reverse transcriptase activity. The second and more recent wave of interest in the retroviral reverse transcriptase relates, of course, to its involvement in the AIDS epidemic.

Although reverse transcriptases are best known for their role in the retrovirus life cycle, it has become clear that analogous enzymes are found in a variety of cell types with diverse functions and phenotypes. Thus, the reverse transcriptase family of DNA polymerases participates in the replication of retrotransposons in several eucaryotic organisms, including yeast (Ty) (5), *Drosophila* (6, 7), and human cells (line elements) (8). A reverse transcriptase, poorly characterized biochemically, synthesizes branched DNA molecules in bacteria (9). In eucaryotes, there are strong arguments supporting the idea that RTs play a role in the emergence of pseudogenes and Alu repeat sequences (10), as well as in the splicing of some mitochondrial introns (11) and the transfer of genetic information from mitochondria and chloroplasts to the nucleus (12, 13). Moreover, telomerase, an enzyme that adds a small repetitive DNA sequence to the ends of chromosomes, can be regarded as a special type of reverse transcriptase (RT) (14).

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[2]Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV-1, human immunodeficiency virus type 1; AZT, azidothymidine; DDI, dideoxyinosine; RT, reverse transcriptase; PBS, primer binding site; cDNA, complementary DNA.

0892-6638/94/0008-0497/$01.50.

Reverse transcriptase has become an excellent target for attempts to arrest the proliferation of HIV-1 for several reasons: *1)* it is a crucial enzyme in the viral replication cycle; *2)* its properties are quite different from those of the other cellular DNA polymerases; and *3)* it is active in the cytoplasmic compartment of the infected cell, separate from the nuclear and mitochondrial DNA polymerases.

This enzyme has therefore become a promising target for inhibiting HIV-1 replication. Nowadays, the therapeutic agents used most frequently in clinics against AIDS are zidovudine, or azidothymidine (AZT) and dideoxyinosine (DDI). These drugs are nucleoside analogs that act as chain terminators of DNA polymerization by HIV-1 RT (for a review, see ref 15).

SYNTHESIS OF RETROVIRAL REVERSE TRANSCRIPTASE

The *gag*, *pol*, and *env* genes are common elements in the organization of all retroviral genomes. In all retroviruses studied to date, two enzymes are found within the infecting virion that are involved in the synthesis and nuclear integration of the proviral DNA. These two enzymes, reverse transcriptase and integrase, are encoded by the *pol* gene, which is located downstream of the *gag* gene. They are synthesized as part of the 160-kDa GAG-POL polyprotein. Upstream of the reverse transcriptase reading frame, the gene *pol* encodes a viral protease, whereas downstream of the reverse transcriptase reading frame the information for the integrase protein is found. The coding region of the *pol* gene has no independent initiation codon; as expected, most ribosomes engaged in the synthesis of the GAG protein terminate protein elongation at the *gag* stop codon. However, a small fraction of these ribosomes continue protein synthesis beyond this termination codon as a result of a translation suppression or frameshifting mechanism, thereby generating a GAG-POL fusion polyprotein (for a recent review, see ref 16). During virion maturation, the GAG-POL polyprotein is cleaved by the virally encoded protease to yield the mature form of both the reverse transcriptase and the integrase proteins. Although the structure and mechanism of action of the retroviral protease have been studied in detail, many questions concerning the structure and function of reverse transcriptase in the polyprotein GAG-POL precursor remain to be answered (17).

FUNCTIONAL FEATURES OF HIV-1 REVERSE TRANSCRIPTASE

Three distinct enzymatic activities have been associated with the retroviral reverse transcriptase (18): *1)* an RNA-dependent DNA polymerase activity involved in the synthesis of the minus strand of the proviral DNA; *2)* a DNA-dependent DNA polymerase activity that catalyzes the synthesis of the plus DNA strand; and *3)* an RNase H activity. This nuclease activity degrades the RNA portion of the RNA-DNA hybrid, generating the RNA primer used for the synthesis of the plus DNA strand. The RNase H activity is also involved in the final removal of the tRNA primer used to initiate first strand synthesis (see below).

It has recently been proposed that HIV-1 reverse transcriptase also possesses a ribonuclease activity that specifically degrades double-stranded RNA (19). However, the existence of such an activity remains controversial and other investigators have suggested that this nuclease activity can be correlated with traces of *Escherichia coli* RNase III that contaminate these bacterially expressed preparations (20).

Genomic RNA from retroviruses is copied into complementary DNA (cDNA) by reverse transcriptase. As for all DNA polymerases, reverse transcriptase needs a primer carrying a free 3' -OH group to initiate cDNA synthesis. The in vivo primer for retroviral first (minus) strand DNA synthesis is a specific tRNA molecule that is selected from total host tRNAs. Avian retrovirus reverse transcriptase forms a stable and specific complex with primer $tRNA^{Trp}$ (for a review, see ref 21). Several experimental approaches have shown that reverse transcriptase is involved in selecting and annealing the primer to the region of the retroviral genome complementary to the 3' terminal 18 nucleotides of the tRNA (primer binding site or PBS). It was deduced from the sequence of the PBS region of HIV-1 RNA that the host tRNA serving as the primer for HIV-1 reverse transcriptase is human $tRNA^{Lys3}$ (22). Indeed, using different experimental approaches including UV cross-linking, RNase footprinting, and gel retardation, a complex between mammalian $tRNA^{Lys3}$ and HIV-1 reverse transcriptase has been demonstrated. The regions of the primer tRNA in close contact with the enzyme have been identified as the anticodon region and the dihydrouridine loop (23, 24).

It has been proposed that the nucleocapsid protein NCp15, a small basic protein derived from the carboxyl-terminal domain of the GAG protein that is crucial for dimerization of the retroviral genome (25), is also involved in annealing the primer tRNA to the PBS region of HIV-1 RNA (26). However, it has recently been shown that only reverse transcriptase is required for the in vitro annealing of primer tRNA to the PBS region, as a tRNA/PBS complex, competent for initiation of cDNA synthesis, can be observed in the absence of NCp15 (27, 28). Nevertheless, NCp15 may be required to improve the tRNA/PBS interaction as synergistic complex formation between primer tRNA and PBS has been observed when both reverse transcriptase and NCp15 are present in vitro. The highly abundant NCp15 protein may therefore play an essential role in viral replication by increasing the efficiency and speed of viral genome replication.

It is apparent that HIV-1 reverse transcriptase is a very flexible protein that may be related to the several functions catalyzed by the enzyme. Studies of the interaction between HIV-1 reverse transcriptase and its primer tRNA support this idea. Thus, important conformational changes of the enzyme in the presence or absence of $tRNA^{Lys}$ have been described (29). These conformational changes induced by primer tRNA affect not only the DNA polymerase activity but also the RNase H activity of the retroviral polymerase (30).

A better understanding of the molecular structure of the interactions between HIV-1 RT and the primer and template emerged from studies of crystals at 7 Å resolution of a ternary complex containing the enzyme, DNA, and an Fab fragment (31). The description of HIV-1 RT complexed with the nonnucleoside Nevirapine at 3.5 Å resolution (32), as well as the recent determination of the ternary structure mentioned above at 3.0 Å resolution (33), has provided a wealth of molecular information about the enzyme (see below in **Fig. 1**).

Biochemical studies have indicated that, in accordance with a general model of primer/template interaction with several DNA polymerases, the 3' end of the oligonucleotide makes a crucial contribution to the binding of DNA polymerases with primers (34, 35). Of all DNA polymerases studied (including avian myeloblastosis virus, AMV, reverse transcriptase), only HIV-1 reverse transcriptase showed

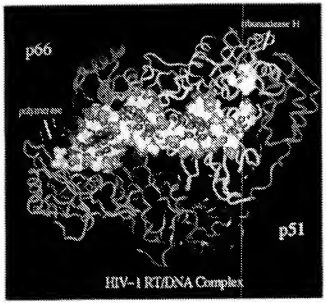

Figure 1. Three-dimensional structure of HIV-1 reverse transcriptase complexed with a double-stranded DNA primer-template based on the 3 Å resolution crystal structure determination described in ref 33. The polypeptide backbone for the HIV-1 RT p66/p51 heterodimer is represented as a solid ribbon trace with subdomains colored as: fingers (blue), palm (red), thumb (green), connection (yellow), and RNase H (orange). The 18-mer/19-mer DNA primer-template is represented as a CPK space-filled model. The DNA has A-form geometry near the polymerase active site, B-form near the RNase H active site, and has a 40–45° bend at the junction of the two regions. Also indicated are the locations of the polymerase and RNase H active sites with space-filling representation of the key acidic residues implicated in catalysis. The separation of the two active sites corresponds to approximately 18 nucleotides in this complex. Figure courtesy of Birgit Roy and Dr. Edward Arnold.

higher affinity to riboprimers than to deoxyriboprimers (36). The use of cross-linking by UV irradiation or platinum ions to create a covalent link between the enzyme and primer allowed the isolation of specific peptides carrying the labeled probe (37). A more "physiological" approach has been attempted in our laboratory. A primer analog with a 5'-end modification was covalently attached to reverse transcriptase. In the presence of the complementary template and a labeled precursor, the elongation of the RT-linked primer by one radioactive nucleotide residue was obtained and the products were visualized by autoradiography. This reaction, due to the "catalytic competence" of the enzyme, readily demonstrates that the 3' end of the primer is localized at or near the catalytic center (38). The specificity of this primer binding reaction was further confirmed by the observation that $tRNA^{Lys}$, the natural primer of HIV-1 RT, can compete for binding of the covalent primer-template to the enzyme. Unexpectedly, this affinity labeling can also be observed when HIV-1 RT was modified by the primer analog in the absence of template. In the case of other procaryotic and eucaryotic DNA polymerases, no primer binding domain labeling was detected in the absence of a template having complementarity with the primer analog (39). These results suggest that competitive modification of the primer binding domain by primer analogs in the absence of template is a feature unique to HIV-1 reverse transcriptase. This is consistent with the results from several groups, in which HIV-1 reverse transcriptase was shown to be capable of binding the primer chain before binding the template (34, 35, 38). This observation may be related to the utilization by the retroviral polymerase of the tRNA as a natural primer. In contrast with other cellular DNA polymerases, which initiate DNA synthesis from short RNA primers synthesized by DNA primase, reverse transcriptase must select its specific primer from the total population of host cellular tRNAs, possibly making this the rate-limiting step in the assembly of an initiation complex. The use of tRNA analogs as primers for the DNA synthesis reaction catalyzed by HIV-1 reverse transcriptase should be an interesting tool with which to assess this hypothesis.

The polymerase and RNase H domains of HIV reverse transcriptase reside within the same protein molecule. It can be assumed that both activities, DNA synthesis and hydrolysis of the RNA hybridized to the newly synthesized DNA, are tightly coupled (40). Modeling based on the crystal structure of HIV-1 RT complexed with an inhibitor predicted that 20 base pairs of an RNA-DNA duplex can be accommodated between the polymerase and RNase H active sites (32). The three-dimensional structure of HIV-1 RT complexed with dsDNA primer-template revealed a separation of 18 nucleotides between the active sites (33). These structural results are consistent with the separation indicated from independent biochemical studies (for references, see ref 41). This spatial separation may be crucial during the retroviral replication cycle. As stated above, the minus strand of proviral DNA is primed by a $tRNA^{Lys}$ of cellular origin, which hybridizes to the 18 nucleotides of the PBS region near the 5' end of HIV-1 RNA. During synthesis of the plus strand of DNA, this primer RNA is removed by the RNase H activity of HIV-1 reverse transcriptase (42). Specific cleavage to release primer tRNA could be guided by the distance between the DNA polymerase active site and the RNase H active site 18 nucleotides downstream. The mechanism of initiation of second strand synthesis may also require that a specific distance be maintained between the two active sites of the enzyme. After synthesis of the first DNA strand, the genomic retroviral RNA template is cleaved into multiple fragments that could potentially serve as primers for plus DNA strand synthesis. However, only one of these, a 19-base RNA primer with a purine-rich sequence, is actually used by the reverse transcriptase. This length may be related to the observed distance, mentioned above, of 18–20 nucleotides between the two active sites.

STRUCTURE OF HIV-1 REVERSE TRANSCRIPTASE

HIV-1 reverse transcriptase is a heterodimer of 66 kDa and 51 kDa (usually referred as p66 and p51, respectively). The p51 polypeptide is derived from the proteolytic maturation of the p66 precursor by the retrovirally encoded protease, which cleaves the transcriptase, at the carboxyl terminus of one of the subunits. Assuming that the cleavage is performed at the homodimer level, a delicate mechanism of proteolysis must be involved in the maturation of the p66/p66 precursor homodimer to the biologically active p66/p51 polymerase. The p66 subunit has the DNA polymerase and RNase H activities, and is considered to be the catalytic core of the enzyme. These activities have been predicted and experimentally demonstrated to reside in separate domains: the amino-terminal p51 domain carries the DNA polymerase activity and the 15-kDa carboxyl-terminal fragment the RNase H activity. This functional separation is a general attribute of all retroviral reverse transcriptases and has been especially well defined in the case of the murine reverse transcriptase

(43). However, a detailed linker insertion mutagenesis study showed that this separation is less clear for the HIV-1 reverse transcriptase, as the RNase H activity is affected by mutations in the DNA polymerase domain (44). The DNA polymerase and RNase H enzymatic activities described above are found in the p66 subunit, whereas p51 is devoid of RNase H activity. It was suggested that p51 had no DNA polymerase activity, but several laboratories have shown that depending on the in vitro assay conditions, p51/p51 recombinant forms can display significant activity in DNA synthesis (44–47). On the other hand, reports that the virion-purified or recombinant p15 peptide have RNase H activity have been contradicted. However, the in vitro reconstitution of p51 and p15 led to significant recovery of RNase H activity (46). The precise role of p51 in the virion active polymerase remains to be determined as it seems to be a "silent" subunit with respect to DNA polymerase activity (45). In most cases, recombinant HIV-1 reverse transcriptase is isolated as a dimer. There is convincing experimental evidence that the affinity values between subunits in the p66/p51 heterodimer are higher than in the homodimers p66/p66 and p51/p51. Moreover, dimerization of the retroviral DNA polymerase seems an absolute requirement for both the DNA polymerase and RNase H activities (47, 48). These results have led to speculations that subunit dimerization may be an interesting target for chemotherapeutic intervention. The 3.0 Å resolution structure of the HIV-1 RT/DNA complex with complete amino acid assignments for the enzyme makes it possible to detail the molecular interactions at the interface between the p66 and p51 subunits (33) (Fig. 1). The availability of the coordinates for that structure (through the Brookhaven Protein Databank: entry PDBIHMI) describing the location of all the amino acids in HIV-1 RT permits inspection of the residues that form the dimer interface.

Recently published three-dimensional structural studies of HIV-1 RT are making major contributions to furthering our understanding of structure-function relationships of this enzyme. One of the first approaches that provided structural information about HIV-1 reverse transcriptase was based on experimental data coming from neutron small-angle scattering data. This approach gave the rough spatial arrangement of the p51 and p66 subunits and the position of the RNase H-containing domain, p15 (49). Using the techniques of X-ray crystallography, two groups have succeeded in describing the three-dimensional structure of HIV-1 reverse transcriptase (for a recent review, see ref 50). The group headed by T. Steitz (32) at Yale has reported the detailed structure of HIV-1 reverse transcriptase complexed with the non-nucleoside inhibitor Nevirapine at 3.5 Å resolution. Recently the laboratories of Arnold and Hughes have refined their previously described structure (31) at 7 Å resolution of a ternary complex of HIV-1 reverse transcriptase, a monoclonal antibody Fab fragment and a double-stranded DNA template-primer. They have now determined the crystal structure of the ternary complex at 3 Å resolution using an 18-base/19-base double-stranded DNA primer-template (33). The overall structure is similar to that described by Kohlstaedt et al. (32); the DNA template-primer complexed to the enzyme has A-form and B-form regions separated by a significant bend (33).

The structure described by these groups reveals an unprecedented degree of asymmetry in the heterodimer (32, 33). The polymerase domain of the p66 subunit has a large cleft analogous to that of the Klenow fragment of *E. coli* DNA polymerase I. This cleft may correspond to the primer-template site. The p51 subunit, which is identical to p66 except for the p15 domain, has a very different structure and does not show a similar cleft. The formation of a recombinant p51/p51 form, active in cDNA synthesis (45), may be related to a structural change associated with the formation of a cleft capable of accommodating the primer-template complex. Kohlstaedt et al. (32) have named the different subdomains of the structure as fingers, palm, and thumb in light of the striking anatomical resemblance to that of a right hand. This structural similarity between reverse transcriptase and the *E. coli* polymerase is quite remarkable, given the lack of significant sequence homology between these two enzymes. This overall picture suggests that the general structure of key domains of all DNA polymerases, such as the primer-template, dNTPs, or divalent metal binding regions, could be structurally conserved. The high-resolution structure of HIV-1 RT/DNA complex has permitted Arnold et al. (33) to propose the specific functions of conserved elements located near the polymerase active site. This is the first structure of any polymerase in which nucleic acid has been bound to the polymerase active site in a mode relevant to polymerization (Fig. 1). In particular, they observed that several structural motifs conserved in all RNA-dependent polymerases appear to function as a clamp to hold the primer-template in a precise geometry relative to the active site such that efficient polymerization can occur. These elements, located primarily in the palm subdomain of p66, were designated as "primer grip" and "template grip" as they interact strongly with the primer and template strands, respectively. The same group proposed that two α-helices of the p66 thumb may function as "tracks" over which the primer and template strands may roll during processive DNA synthesis. As additional structures including bound dNTPs and different primer-templates are determined, it may be possible to ultimately obtain a frame-by-frame structural description of DNA polymerization.

The X-ray structure of the RNase H domain has been determined separately to a resolution of 2.4 Å (51). The RNase H domain is very similar in overall structure to *E. coli* RNase H, with two divalent metal cations necessary for catalysis located near a cluster of four acidic amino acids residues (Asp^{443}, Glu^{478}, Asp^{498}, and Asp^{549}). The most important application of the crystallographic structural data of HIV-1 reverse transcriptase may be in the rational design of specific inhibitors for therapeutic use.

ENZYME INHIBITORS AND THERAPEUTIC AGENTS

For those working in the field of DNA polymerases, the availability of specific inhibitors has been of great help in the characterization of these enzymes. For instance, inhibitors such as aphidicolin, butylphenyldGTP, and aracytosine have been extremely useful in studying the roles of replicative cellular DNA polymerases. The emergence of AIDS and the fundamental role played by RT in retroviral replication have made this enzyme a key target in the search for specific inhibitors, as these drugs may become powerful therapeutic agents in addition to being useful tools for the biochemical analysis of the enzyme. As mentioned at the beginning of this review, the HIV-1 RT inhibitors AZT and DDI are extensively used in clinical efforts to retard pathological symptoms associated with AIDS. As it would be impossible to summarize in this short review the considerable amount of information gathered in recent years on the inhibitors of HIV-1 reverse transcriptase, we will give a brief survey of the three main families of compounds that can inhibit reverse transcriptase (Fig. 2).

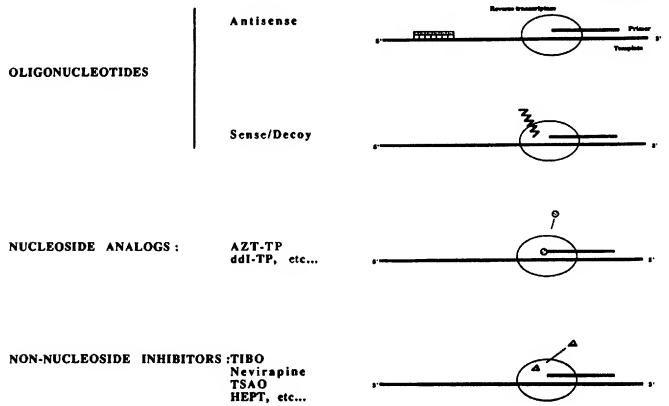

Figure 2. Reverse transcriptase inhibitors that are potential therapeutic agents against HIV-1. antisense oligonucleotides, (▭); sense (decoy) oligonucleotides, (∿); nucleoside analogs, (⊖); non-nucleoside inhibitors, (▲).

Oligonucleotides

Oligonucleotides can inhibit DNA synthesis by HIV-1 reverse transcriptase via a sense or an antisense strategy. Sense oligonucleotides directly inhibit HIV-1 reverse transcriptase, as in the case of the phosphorothioate derivative polydeoxynucleotide SdC_{28}, which is a strong inhibitor of the enzyme (35). The exquisite sensitivity of the viral polymerase to oligonucleotides may be related to the unexpected inhibition of DNA synthesis we have observed by tRNA and tRNA fragments, when these primer molecules are free and not annealed to the PBS (52). The possibility of targeting the active center of the enzyme with substrates or substrate-like compounds has also been elegantly exploited by using derivatives of phosphorothioate and dithioate oligos that correspond to portions of the tRNA molecule that interact with the enzyme. The high affinity and the strong degree of inhibition displayed by these modified oligonucleotides encourage further studies for their use as a potential new family of therapeutic agents (53).

The antisense strategy, based on the formation of a stable complementary duplex, is widely used to arrest gene expression in eucaryotic cells (for a review, see ref 54). Although no clinical trials have yet been performed with these types of agents, strong inhibition has been observed when incubating HIV-1-infected cells with oligodeoxynucleotides complementary to specific regions of the HIV-1 genome (55). In vitro reverse transcription by HIV-1 polymerase is inhibited by complementary oligodeoxynucleotides (56). Similar results were obtained using avian and murine reverse transcriptases (57). The RNase H activity associated with HIV-1 RT is not always necessary for this inhibition, as the synthesis of cDNA was equally inhibited by some antisense oligonucleotides when an HIV-1 reverse transcriptase devoid of this activity was used. However, in some cases the RNase H activity was clearly involved in the in vitro inhibitory blocking effect of antisense oligonucleotides (56, 57).

Nucleoside analogs

Nucleoside analogs, like AZT and DDI, are chain terminators and thus arrest cDNA synthesis by HIV-1 reverse transcriptase after being incorporated into the nascent DNA chain (15).

DNA polymerases α and β, the two major host DNA polymerases, are able to incorporate azidothymidine and dideoxynucleosidetriphosphates. Although retroviral replication takes place, most probably, in the nucleocapsid structure in the cytoplasm and the human polymerases are confined to the nucleus, these results may be related to the secondary effect of these nucleoside analogs. The incorporation of AZT by DNA polymerase α is lower than in the case of HIV-1 RT whereas ddCTP is not incorporated at all. However, the behavior with AZT depends on whether the α enzyme is in the processive step of DNA elongation or during the initial recognition of the primer 3' end. In the latter case, the α polymerase is able to incorporate one AZT residue. Polymerase β is able to incorporate AZT and ddCTP in both steps of DNA synthesis, although at a lower extent than HIV-1 reverse transcriptase (58). As pointed out by these

authors, these results raise the concern that the behavior of these polymerases may cause adverse effects on human metabolism and may induce mutations in patients under long-term treatment with this nucleoside analog. More recently, the same authors showed that 4' azydothymidine, an AZT analog that retains the 3' OH group, does not act as a chain terminator in human T-cells. This analog very strongly inhibited HIV-1 reverse transcriptase whereas the affinity toward host DNA polymerases was very low (59).

Non-nucleoside compounds

Non-nucleoside compounds, which have been found by routine screening, affect the DNA polymerase activity of HIV-1 reverse transcriptase by mechanisms that are not yet understood. Some of these compounds affect HIV-1 reverse transcriptase without inhibiting the HIV-2 polymerase. The HIV-1 reverse transcriptase structure determined by Steitz et al. (32) contains Nevirapine (60), which is a non-nucleoside compound able to act as a noncompetitive inhibitor of HIV-1. This drug binds at a site of the palm and thumb subdomains distal to the substrate-binding pocket. The Nevirapine molecule is placed near the expected primer binding end in the p66 subunit and seems to interact with the residues Tyr^{181} and Tyr^{188} as predicted from previous biochemical studies showing cross-linking to these conserved residues, which are part of the conserved (183–186) Y-M-D-D sequence.

An aspect of HIV-1 reverse transcriptase function that has not been discussed in this review is the fidelity of this enzyme. It is well documented that RT possesses a low degree of fidelity during DNA polymerization. The misincorporation level ($1–2 \times 10^{-4}$ per nucleotide per cycle), although not significantly different from that of avian and murine reverse transcriptases, is significantly higher when compared with the replicative cellular DNA polymerases, whose error level is on the order of 10^{-9} to 10^{-11} mutations per base pair per replicative cycle (61).

As is suggested above, structural data are crucial for the design of new inhibitors of HIV-1 RT. The problem of inhibitor design is exacerbated, however, by the rapid accumulation of genomic mutations. The effectiveness of AZT or ddI as chain terminators is limited, as resistance to these substances appears after several months of clinical treatment. There is no doubt, based on the contributions of the Larder laboratory (62), that alterations in the RT polymerase domain confer resistance to inhibition. Unfortunately, non-nucleoside analogs like Nevirapine also frequently lead to genomic mutations affecting the two interacting tyrosines, which rapidly reduce the effectiveness of these drugs. Mutations of HIV-1 reverse transcriptase that lead to the emergence of enzymes resistant to inhibitors are thus found in the case of both nucleoside analogs and non-nucleoside inhibitors.

It is extremely important to determine further structural details of HIV-1 reverse transcriptase with its substrates and inhibitors to facilitate the emergence of more potent and selective inhibitors at sites that may be conserved for enzymatic activity. FJ

The authors hope that their colleagues will understand that due to the limited length allowed for this review, the names of many researchers who have made important contributions in the field of HIV-1 reverse transcriptase could not be mentioned directly. We are very grateful to Dr. E. Arnold for providing an illustration for this review and for his suggestions concerning the manuscript. The authors are indebted to the Editor, an anonymous referee, and Dr. David Julius (Department of Pharmacology, USCF, San Francisco, CA, USA) for their help in improving the manuscript. Work in the authors' laboratory has been supported by the French Agency for Research Against AIDS (ANRS), the Association de Recherches contre le Cancer (ARC), the CNRS, the University of Bordeaux II, and the Conseil Régional d'Aquitaine.

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Exhibit 2: Nugent et al., "The Telomerase Reverse Transcriptase: Components and Regulation," *Genes & Development* 12:1073-1085 (1998)

REVIEW

The telomerase reverse transcriptase: components and regulation

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The idea that chromosomes have special terminal structures first arose as a consequence of experiments conducted by Muller, who found that treatment of *Drosophila* with X-rays rarely resulted in terminal deletions or inversions of the chromosomes (Muller 1938). Complementary experiments in maize by McClintock expanded upon the idea that telomeres, the physical ends of chromosomes, are required for chromosome stability, by contrasting the breakage-fusion-bridge cycle resulting from broken dicentric chromosomes with the stability of chromosomes with intact termini (McClintock 1941, 1942). With the dawn of molecular biology, telomeres in most eukaryotes are now known to be composed of short repeated G-rich sequences complexed with proteins to form a special heterochromatin-like structure. More recent experimental manipulation of chromosome termini and of the proteins that bind them have confirmed the early observations of Muller and McClintock, showing that a primary role of telomeres is to insulate the ends of chromosomes both from fusion with other ends and from nucleolytic digestion (Counter et al. 1992; Sandell and Zakian 1993; Garvik et al. 1995; van Steensel et al. 1998).

Not only do telomeres function as protective caps at the ends of chromosomes, but they also facilitate the complete replication of chromosomes. Conventional DNA replication machinery utilizes an RNA primer to initiate DNA synthesis, leading to the problem that extreme terminal sequences will not be represented on the 5′ end of one daughter DNA strand, after removal of a terminal RNA primer. Without a mechanism to replenish these sequences, chromosomes will inevitably shorten as they proceed through successive divisions and, at some point, the ends of chromosomes will be too short to continue to provide the capping function necessary for maintaining genomic stability. The solution to this end-replication problem that has been adopted by most organisms is to use a telomere-specific DNA polymerase called telomerase that extends the 3′ end of the G-rich strand of the telomere. The synthesis of telomeric DNA by telomerase thereby serves to counter the consequence of semiconservative DNA replication.

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Telomerase was first identified biochemically >12 years ago (Greider and Blackburn 1985) and shown to use an extraordinary mode of synthesis, relying on an intrinsic RNA to serve as a template for the polymerization of the telomeric DNA sequences (Greider and Blackburn 1989; Yu et al. 1990). In the last few years, there has been a substantial advance in our understanding of the additional subunits of telomerase. This review discusses the recent identification and characterization of the reverse transcriptase catalytic component, evaluates the role(s) of other potential protein subunits in telomerase activity or regulation, and concludes with a consideration of the consequences for the cell when telomerase is absent. Regulation of telomerase by the telomere has been the subject of several recent excellent reviews (Brun et al. 1997; Shore 1997) and will not be discussed here.

The RNA subunit of telomerase

Unlike other polymerases responsible for replication of genomic DNA, telomerase activity depends on an essential RNA subunit. Telomerase was first shown to be an RNA-dependent DNA polymerase by characterization of the enzyme isolated from the unicellular ciliate *Tetrahymena*. The identification of a 159-nucleotide RNA component containing the sequence 5′-CAACCCCAA-3′, complementary to the $d(TTGGGG)_n$ telomeric repeat synthesized by *Tetrahymena* telomerase, suggested that this region of the RNA provides a template for telomere synthesis (Greider and Blackburn 1989). Mutational analysis of the CAACCCCAA sequence resulted in an enzyme that synthesized altered telomeric repeats, confirming the templating hypothesis (Yu et al. 1990; Yu and Blackburn 1991). Since the first discovery in *Tetrahymena*, telomerase has now been identified from a wide variety of sources and in every case it has been shown to be a ribonucleoprotein (RNP) complex, with the information that dictates the sequence of the telomere contained within the RNA component.

The templating region of the telomerase RNA can be dissected into two functionally separable subdomains, employed in primer alignment and primer extension (Autexier and Greider 1994, 1995; Gilley and Blackburn 1996). The contributions of different residues of the template to these two functions have been dissected in detail

with the *Tetrahymena* telomerase RNA. These experiments have shown that one end of the RNA template (3'-AAC-5'; see Fig. 1; Table 1) serves to align the telomeric DNA primer for the extension step, via basepairing between the 3' terminus of the primer and a portion of the template. Subsequent elongation occurs by copying the remaining six residues of the template onto this telomeric end. These first round products can be further elongated if the new telomeric terminus is translocated back to the primer alignment site, so that the primer is repositioned for another round of synthesis. Although the *Tetrahymena* enzyme is capable of multiple rounds of elongation from the same telomeric primer (Greider 1991), a processive mode of elongation is not exhibited by all telomerases (Prowse et al. 1993; Prescott and Blackburn 1997a). Processivity is dictated by more than base pairing interactions at the primer alignment site; telomerase can also interact with telomeric substrates at a second, RNA-independent, primer binding site, called the anchor site, that contributes to processive elongation (Morin 1989, 1991; Harrington and Greider 1991; Collins and Greider 1993; Lee and Blackburn 1993; Melek et al. 1996). Telomerase protein(s) that contribute to anchor site function are discussed in subsequent sections.

Although most telomerases copy their template region faithfully, resulting in telomeres composed of invariant repeats of the same telomeric sequence, a minority of telomerase enzymes synthesize telomeres with an irregular sequence composition (Table 1). This can be attributable to a high frequency of dNTP misincorporation, as appears to be the case for some *Paramecium* enzymes (McCormick-Graham et al. 1997), or can be the consequence of stuttering across the template by the enzyme, whereby partial translocation during the synthesis of a single repeat causes duplicate copying of one or more nucleotides of the template (Yu and Blackburn 1991; Cohn and Blackburn 1995). *Saccharomyces cerevisiae* telomeres exhibit extreme sequence variability, consisting of an irregular repeat conforming to the consensus $T(G)_{2-3}(TG)_{1-6}$ (Shampay et al. 1984; Wang and Zakian 1990). This follows from the possibility of variable positioning of the telomeric primer along the template (Kramer and Haber 1993; Prescott and Blackburn 1997a). Whether the imprecision of these telomerases can be attributed solely to features inherent in the RNA subunit is not clear. For example, although the *S. cerevisiae* enzyme has a long template compared to that of many other telomerases (Table 1), other budding yeasts with even longer template domains synthesize telomeres composed of regular repeats (McEachern and Blackburn 1994, 1995).

Although the RNA performs a highly conserved function that is central to the polymerization mechanism, there is very little conservation at the primary sequence level among the telomerase RNAs cloned from >25 different species. For example, telomerase RNAs from the hypotrichous versus the tetrahymenine ciliates are so diverged that, with the exception of the template region and an adjacent short region, the primary sequences cannot be aligned readily (Lingner et al. 1994), and even the murine and human RNA subunits show only 65% sequence identity (Blasco et al. 1995; Feng et al. 1995). Despite divergence of the primary sequence of the RNA subunit, the secondary structure has apparently been more conserved; phylogenetic sequence comparison of telomerase RNAs from evolutionarily distant ciliates has led to several related secondary structure proposals (Romero and Blackburn 1991; ten Dam et al. 1991; Lingner et al. 1994; McCormick-Graham and Romero 1996). A common feature of these models is the proposal that the template region is present as an unpaired region of RNA, consistent with the expectation that it is accessible for the polymerization reaction (Fig. 1). Additional structural features include a highly conserved stem I, thought to establish the superstructure of the RNA, a pseudoknot and a set of stem-loop structures that could

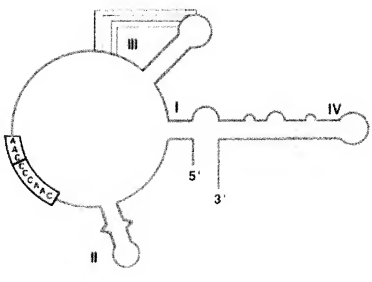

Figure 1. Secondary structure model for telomerase RNA. This representation of a minimal secondary structure for telomerase RNA was proposed from comparative sequence analysis of a number of *Tetrahymena* species (Romero and Blackburn 1991; ten Dam et al. 1991). The potential pseudoknot structure involving stem-loop III is indicated by the straight lines (*top*). The three boxed residues AAC (3' 5') align the telomeric end, and the following six boxed residues CCCAAC (3'-5') template nucleotide addition. Helix II is not found in other ciliate RNAs (Lingner et al. 1994), and a novel fifth helix is present between helices I and III in *Paramecium* RNAs (McCormick-Graham and Romero 1996).

Table 1. *A comparison of the sequence of the RNA template region and the corresponding telomeric repeat synthesized by six different telomerases*

Organism	RNA Template (3′–5′)	Telomeric Repeats (5′–3′)	Reference
Tetrahymena	AACCCCAAC	$(GGGTTG)_n$	Greider and Blackburn (1989)
H. sapiens	CAATCCCAATC	$(GGGTTA)_n$	Feng et al. (1995)
M. musculus	TCCCAATC	$(GGGTTA)_n$	Blasco et al. (1995)
Paramecium	ACCCCAAC	(GGGTTG or GGTTTG)$_n$	McCormick-Graham and Romero (1996)
S. cerevisiae	ACACACACCCACACCAC	$[TG)_{6-8}(TG)_{1-6}]_n$	Singer and Gottschling (1994)
K. lactis	AAACTAATCCATACACCACATGCCTAAACT	$(ACGGATTTGATTAGGTATGTGGTGT)_n$	McEachern and Blackburn (1995)

The RNA template is shown 3′ to 5′, for ease of comparison with the telomeric repeat synthesized by the respective enzyme. Although the *Tetrahymena* telomeric repeats are conventionally written $(GGGGTT)_n$, they are shown here as the permutation $(GGGTTG)_n$ to emphasize the order of synthesis of these six nucleotides by the templating residues after alignment of the primer. The residues of the *Tetrahymena* template that are used for substrate alignment are underlined. The sequences of the template regions of other telomerases are derived from sequence inspection of the RNA and comparison to the telomeric repeat synthesized by the respective enzymes. However, the precise 5′ and 3′ boundaries of these additional templates have not yet been determined (although the templates of the *S. cerevisiae* and *Paramecium* enzymes have been investigated in some detail by mutagenesis; Prescott and Blackburn 1997b; McCormick-Graham et al. 1997). For example, it is not clear whether the 3-nucleotide difference between the length of the proposed mouse and human templates represents a functional difference, nor has it been shown that all of the nucleotides 3′-CAAT-5′ in the human RNA are utilized in substrate alignment. dNTP misincorporation at the residue indicated in boldface type in the *Paramecium* template has been proposed to account for the mixture of TTTGGG and TTGGGG repeats observed in vivo in *Paramecium tetraurelia* telomeres (McCormick-Graham et al. 1997).

contribute to telomerase assembly and/or function by providing potential telomerase protein binding domains. Probing the native RNA structure through chemical modification of the *Tetrahymena* telomerase RNP complex has provided data consistent with the proposed structural model, although the structure of the naked RNA appears to be somewhat different (Battacharyya and Blackburn 1994; Zaug and Cech 1995). Functional support for a conserved RNA structure has also come from cross-species swap experiments, using RNAs that are 25%–50% divergent: Chimeric telomerases assembled in vivo with proteins from one species and the telomerase RNA from another are still capable of synthesizing telomeric repeats (Bhattacharyya and Blackburn 1997; McCormick-Graham et al. 1997).

One caveat to the model shown in Figure 1 is that it is a static structure, which contrasts with the conformational change that presumably occurs in response to repositioning of the primer relative to the template as telomeric DNA synthesis proceeds. Lingner et al. (1994) have proposed that a conversion between the conserved pseudoknot and the stem–loop III structure could serve as a conformational switch, and structural probing data are consistent with formation of the pseudoknot when the template is unoccupied (Zaug and Cech 1995). This hypothesis has been tested in vitro using partial reconstitution of the telomerase complex, by nuclease digestion of the native RNA and replacement by in vitro-transcribed telomerase RNAs; in this assay, telomerase reconstituted with RNA mutated to abolish the pseudoknot structure still retains wild-type levels of enzyme activity (Autexier and Greider 1998). This same approach also showed that helices III and IV are similarly dispensable for enzyme catalysis. The conservation of these structures, however, suggests that they may be critical in vivo, perhaps by providing binding sites for proteins that confer essential regulatory functions.

The secondary structure described above has not been extended to distant species, such as the much longer telomerase RNAs from budding yeast (1.3 kb for *S. cerevisiae* and *Kluyveromyces lactis* compared to 147–191 bases for the ciliates). In addition, in vivo observations combined with experimental manipulation indicate that sequences between the template region and the 5′ terminus of the RNA are not essential for mammalian telomerase function. The template domains of the ciliate RNAs are located 35–49 nucleotides from the 5′ end and, consistent with the involvement of the 5′ end in the conserved stem I (Fig. 1), removal of 15 nucleotides from the 5′ terminus of the *Tetrahymena* RNA abolishes telomerase activity (Autexier and Greider 1998). In contrast, although the template sequence of the human RNA is similarly located 45 nucleotides from the 5′ terminus, removal of the first 43 nucleotides only partially reduces human telomerase activity (Autexier et al. 1996). This experimental observation with the human RNA parallels the observation that the natural 5′ terminus of the mouse telomerase RNA occurs only 2 nucleotides before the template (Hinkley et al. 1998). Greider and colleagues have suggested that the variation in template position between the mouse and the human RNA, and the resulting differences in RNA structure, may contribute to the differing degrees of in vitro processivity exhibited by the two enzymes.

Finally, in addition to alignment and templating functions, several observations indicate that the template region of the RNA directly participates in enzyme action by contributing to both the structure and function of the enzyme active site. Several different single base changes in the template of the *Tetrahymena* RNA result in loss of enzyme fidelity: The insertion of a single C residue in the template results in an enzyme that both in vitro and in vivo displays extreme stuttering, adding multiple sequential dG residues, whereas other template mutations

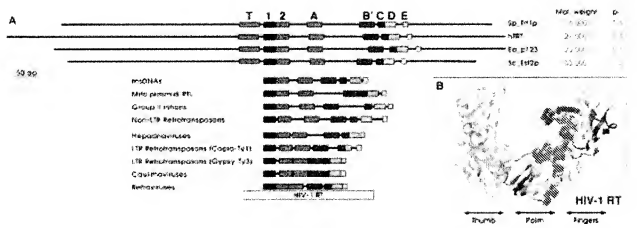

Figure 2. Comparison of protein structural features in telomerase catalytic subunits with other RTs (reprinted, with permission, from Nakamura et al. 1997, American Association for the Advancement of Science). (*A*) The locations of the seven sequence motifs conserved in RTs (1, 2, A, B', C, D, and E, defined by Xiong and Eickbush 1990), are indicated by the colored boxes. The gray box indicates the domain uniquely found in telomerases (motif T, Nakamura et al. 1997). The entire lengths and isoelectric points (pI) of the telomerase proteins are represented; the contributions that domains outside of the RT motifs make to telomerase function have yet to be defined in detail. For the other polymerases, only the region encompassing the *RT* motifs is shown. The open box labeled HIV-1 RT (human immunodeficiency virus type 1) RT indicates the portion of this protein depicted in *B*. (*B*) The crystal structure of the HIV-1 RT p66 subunit is shown as viewed from the back of the right hand (Brookhaven code 1HNV). This structure has been color coded as in *A* to show the spatial arrangement of the RT motifs.

lead to dNTP misincorporation (Yu and Blackburn 1991; Gilley et al. 1995). In addition to these effects on fidelity, template mutations can also result in premature product dissociation (Gilley et al. 1995). Thus, specific residues of the RNA template may collaborate with telomerase protein(s) to form an optimal active site architecture.

The catalytic subunit of telomerase is a reverse transcriptase

The dependence of telomerase polymerase activity upon RNA formally defined the enzyme as a specialized type of reverse transcriptase (RT). During the last year, the discovery of the long-sought-after catalytic component of telomerase revealed that it is in fact a reverse transcriptases, related to known RTs by both amino acid sequence and presumably by evolution. This has provided a key step toward a detailed understanding of the mechanism of chromosomal DNA synthesis by telomerase. Identification of this telomerase protein resulted from convergence of complementary biochemical and genetic strategies in the ciliate *Euplotes aediculatus* and the yeast *S. cerevisiae*. In *Euplotes*, biochemical fractionation of the enzyme identified two telomerase subunits, p123 and p43, that extensively copurify with telomerase activity and are in apparent stoichiometrically equivalent ratios with the RNA subunit (Lingner and Cech 1996). An independent genetic approach recovered four yeast *EST* (ever shorter telomeres) genes that, when mutated, confer a telomere replication phenotype in vivo (Lundblad and Szostak 1989; Lendvay et al. 1996). Comparison of the sequence of the yeast 103-kD Est2 protein with the *Euplotes* p123 subunit revealed that these two proteins are homologs, sharing ~20% sequence identity and more extensive sequence similarity over the length of both proteins. Most notable was the presence in both of a set of motifs common to RTs, marked by the presence of a number of highly conserved residues (Lingner et al. 1997a). A subset of these amino acid sequence motifs had previously been shown to form a conserved protein fold comprising the active site of reverse transcriptases (Kohlstaedt et al. 1992; Arnold et al. 1992; Jacobo-Molina et al. 1993), with three invariant aspartates that are thought to be critical for catalysis (Fig. 2; Larder et al. 1987; Boyer et al. 1994). Single amino acid changes introduced into the comparable aspartates of the Est2 protein abolished yeast telomerase activity in vitro and conferred an in vivo telomere replication defect, demonstrating that these residues are essential for telomerase catalysis (Counter et al. 1997; Lingner et al. 1997a).

Rapid on the heels of the characterization of the telomerase catalytic subunit in *Euplotes* and yeast has been the identification of the same component in *Schizosaccharomyces pombe* (Nakamura et al. 1997) and in humans (Harrington et al. 1997b; Kilian et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997; Nakayama et al. 1998). These proteins, like those from *S. cerevisiae* and *Euplotes*, also show sequence and mechanistic similarity with known RTs, and hence this protein subgroup has been named TERT (telomerase reverse transcriptases).[1] The TERT protein family is most similar in se-

[1]The human protein and gene have been referred to previously as hTRT (telomerase reverse transcriptase) (Nakamura et al. 1997), *hEST2* (Meyerson et al. 1997), hTCS1 (telomerase catalytic subunit 1; Kilian et al. 1997); and TP2 (telomerase protein 2, Harrington et al. 1997b).

quence to RTs such as non-LTR retrotransposons and group II introns that, like telomerase, extend their RNA-templated polymerization from DNA 3′ hydroxyl primers. However, despite the overall similarities with RNA-dependent polymerases, including the three aspartate residues required for enzyme catalysis, telomerases from disparate organisms are more related to one another than to other polymerases and thus appear to form a distinct subgroup (Eickbush 1997; Nakamura et al. 1997). Several features distinguish telomerase RTs, such as a unique region of sequence conservation termed the "T motif," as well as a large amino-terminal basic domain (Lingner et al. 1997a; Nakamura et al. 1997). Compared with known RNA-dependent polymerases, telomerases also appear to have more residues intervening between motifs A and B′. This suggests that the "finger" region of telomerase, a domain in which the HIV RT interacts with its template (Kohlstaedt et al. 1992; Jacobo-Molina et al. 1993), may have functionally distinct properties.

These variations in the features of the telomerase RT motifs may reflect the obvious mechanistic differences between this enzyme and conventional RTs. Whereas RTs are capable of copying long stretches of RNA molecules, the catalytic reaction of telomerase differs in that it is restricted to using only a small portion of its RNA subunit as a template, with the borders of the telomerase RNA template region tightly defined. However, under certain in vitro conditions, the HIV RT can be made to stutter, such that repetitive stretches of the same templated base are synthesized to produce a homopolymeric product (Ricchetti and Buc 1996). Whether this perturbation of the RT mechanism, which depends on extensive primer misalignment, is representative of that normally used by telomerase will require further study; as described above, experimental manipulation of telomerase can also cause stuttering (Yu and Blackburn 1991; Gilley et al. 1995). It will be of substantial interest to compare the tertiary structure of telomerase to that of other RNA-dependent polymerases.

Recent investigations with the human TERT protein have led to several insightful observations; these experiments promise to reveal future details about the core enzyme as well as the in vivo regulation of telomerase in human cells. Most normal cells have undetectable levels of telomerase activity and also fail to express hTERT. Strikingly, enzyme activity in telomerase-negative human cell lines can be restored by the ectopic expression of hTERT (Weinrich et al. 1997; Counter et al. 1998; Nakayama et al. 1998). Thus, the catalytic protein is the only limiting factor for telomerase activation in at least a subset of normal human cells, suggesting that regulation of hTERT expression may be a key target during cellular immortalization. By restoring telomerase activity to normal cells, this technique also has laid the groundwork for assessing whether conversion to telomerase proficiency can reverse the mortal growth characteristics of normal cells; this experiment is discussed in a later section.

A complement to these in vivo experiments is the demonstration that coexpression of the hTERT protein and the human telomerase RNA in reticulocyte lysates is capable of reconstituting enzyme activity (Weinrich et al. 1997; Beattie et al. 1998). This observation suggests that the core enzyme complex may consist of only two components, the RNA and the telomerase reverse transcriptase protein, although in the absence of reconstitution with purified factors, it is not possible to rule out a contribution of additional components from the lysate. However, the availability of this in vitro reconstitution assay has already allowed rapid functional tests of these two components of the core complex, such as dissection of the RNA subunit (Beattie et al. 1998) and the demonstration that the T motif, specific to the telomerase RTs, is required for enzyme activity (Weinrich et al. 1997). An additional consequence is that this in vitro system may accelerate the search for inhibitors of telomerase activity; such inhibitors will be critical in testing the hypothesis that telomerase reactivation plays a role in oncogenesis. Subsequent isolation of derivatives of the core complex that are resistant to such inhibitors could facilitate further the mechanistic dissection of telomerase.

Prior to cloning of any of the TERT proteins, cross-linking studies with *Euplotes* telomerase indicated that the large subunit of the enzyme (presumably corresponding to the p123 catalytic component) contains a second site for telomeric DNA binding, called the anchor site (Hammond et al. 1997). The anchor site had been functionally defined by studies showing that primer recognition and processivity of the telomerase enzyme are influenced by the presence of G-rich telomeric sequences at the 5′ end of the primer, even when the 3′ terminus is nontelomeric (Morin 1989, 1991; Harrington and Greider 1991; Lee and Blackburn 1993; Melek et al. 1996). This site is distinct from the binding that occurs between the 3′ end of the DNA primer and the template region of the RNA. Positioning the 5′ end of the primer in the anchor site is thought to contribute to processivity by preventing dissociation of the primer from the enzyme during translocation on the RNA template of the newly extended 3′ terminus. In studies with the *Euplotes* enzyme, in which the 3′ end of the primer was bound in the active site, cross-links between DNA and protein were localized 20–22 residues from the 3′ end, consistent with the prediction for an anchor site interaction with its primer (Hammond et al. 1997). Intriguingly, the use of partially duplex substrates with 3′ single-strand overhangs, which should resemble natural telomeres more closely, led to cross-links between protein and the duplex portion of the substrate. As expected, cross-links between the catalytic protein subunit and the telomerase RNA were also observed.

Potential additional components of telomerase

It is likely that telomerase, like most other polymerases, will consist of a core enzyme associated with other factors to form a holoenzyme complex. These additional factors may provide critical roles such as recruiting and regulating the interaction of telomerase with the telomere. Other holoenzyme components may modulate en-

zyme activity, such as processivity or the stabilization or dissociation of primer/template interactions. Candidates for additional telomerase subunits discussed in this section have come from several different sources, again relying on both genetic and biochemical approaches.

The first telomerase-associated proteins were identified by biochemical fractionation of telomerase from *Tetrahymena* (Collins et al. 1995). Two proteins, p80 and p95, were recovered by copurification with enzyme activity and the telomerase RNA. Although neither p95 nor p80 appears to encode the catalytic subunit of the *Tetrahymena* enzyme, they do have biochemical properties consistent with roles in recognition of the DNA substrate and interaction with the telomerase RNA (Collins et al. 1995; Gandhi and Collins 1998). Mammalian homologs of p80 have also been identified as telomerase-associated proteins (Harrington et al. 1997a; Nakayama et al. 1997); a mammalian p95 homolog has not been recovered. Antisera to p80 or its homologs can immunoprecipitate telomerase activity (Collins et al. 1995; Harrington et al. 1997a; Nakayama et al. 1997), and the human p80 homolog is in a complex with the hTERT subunit (Harrington et al. 1997b), demonstrating that the p80 protein is associated with telomerase in vivo in disparate organisms. It remains to be determined whether either p80 or p95 is essential for telomerase activity in vivo. In vitro, in the absence of the stringent demonstration that addition of p80 or p95 changes the biochemical properties of a core enzyme complex, the evidence that p95 and p80 are components of telomerase rests on copurification with enzyme activity and the biochemical properties of the individual proteins.

Recent experiments with *Tetrahymena* p80 and p95 proteins purified from *Escherichia coli* have shown that each subunit is independently capable of RNA binding activity, although the complex of both proteins has a substantially greater affinity for RNA (Gandhi and Collins 1998). Although binding is not specific for telomerase RNA, similar attempts to achieve specificity by monitoring the association between a synthetic telomerase RNA and purified telomerase depleted of its endogenous RNA have also not yet succeeded. Cross-linking between p80 and the telomerase RNA can be observed with the purified enzyme (Collins et al. 1995) and the murine p80 homolog can associate with its telomerase RNA, as shown by a three-hybrid interaction (Harrington et al. 1997a), supporting the association of this protein with the telomerase complex. Purified p95 also exhibits specific binding to single-stranded telomeric DNA substrates, in contrast to its nonspecific in vitro RNA binding behavior. The binding preference of p95 for different telomeric DNA substrates roughly correlates with the specificity of primers that telomerase can elongate in vitro, although p95 binding does not exhibit a requirement for a free 3′ end (Gandhi and Collins 1998). Cross-linking between the p95 subunit and telomeric primers has also been detected in the context of the purified endogenous enzyme (Collins et al. 1995). These properties suggest that p95 may play a role in substrate recognition in the holoenzyme, possibly by providing the proposed protein anchor site. However, cross-linking between p95 and telomeric substrates does not depend on an intact enzyme complex, as nuclease treatment that abolishes telomerase activity via digestion of the RNA subunit does not abolish cross-linking. In contrast, complexes between telomerase and telomeric substrates, by assayed gel mobility shifts, require an intact enzyme; consistent with this RNA-dependent interaction, an RNase-sensitive cross-link between a 100-kD *Tetrahymena* telomerase protein and elongated telomeric primers has been observed (Harrington et al. 1995). This raises the possibility that p95 could be a telomerase-associated protein that does not depend on its association with telomerase for telomere DNA binding.

In *S. cerevisiae*, genetic screens for mutants with a telomere replication defect have led not only to the identification of the *EST2* gene, encoding the RT subunit of telomerase, but also to other factors involved in telomere replication (Lundblad and Szostak 1989; Lendvay et al. 1996). The three additional proteins (encoded by *EST1, EST3,* and *EST4/CDC13*) may function as components of the holoenzyme or as potential regulatory factors involved in telomerase function. A separate genetic screen, probing another aspect of telomere function, uncovered *TLC1*, encoding the yeast telomerase RNA subunit (Singer and Gottschling 1994). Strains carrying deletions of *EST1, EST2, EST3,* or *TLC1* exhibit the in vivo phenotypes predicted for a telomerase defect (telomere shortening and progressive loss of viability, termed yeast cellular senescence), and epistasis tests have shown that these four genes act in a single pathway for telomere replication (Lundblad and Szostak 1989; Lendvay et al. 1996; Lingner et al. 1997b). However, despite genetic arguments that the role of these genes is in the telomerase-mediated pathway for telomere replication, only mutations in *EST2* and *TLC1* abolish telomerase activity in an in vitro assay (Cohn and Blackburn 1995; Counter et al. 1997; Lingner et al. 1997a). Extracts prepared from the other *est*$^-$ mutant strains still retain activity at levels roughly comparable to that observed in extracts prepared from a wild-type strain (Cohn and Blackburn 1995; Lingner et al. 1997b). Therefore, the additional *EST* genes cannot encode components of the catalytic core of the enzyme, but this does not exclude the possibility that these genes encode holoenzyme subunits that are critical in vivo but dispensable in vitro. Consistent with potential roles as holoenzyme components, the TLC1 telomerase RNA component can be coimmunoprecipitated with Est1p, Est2p or Est3p (Lin and Zakian 1995; Steiner et al. 1996; Lingner et al. 1997b; T. Hughes and V. Lundblad, unpubl.). However, a substantial caveat to these immunoprecipitation experiments is that the Est proteins are present at such low levels that they cannot be detected in the starting extract, and, as a result, the stoichiometry of each protein relative to the RNA cannot be monitored. Thus, other approaches will be necessary to determine whether these proteins are integral components of the enzyme complex or are more transiently associated with telomerase.

Insight into the role of the Est1 protein has come from analysis of protein partially purified from an *E. coli* expression system, in isolation from other potential telomerase subunits (Virta-Pearlman et al. 1996). Est1 protein binds in vitro specifically to single strand G-rich telomeric DNA, with a requirement for a free 3′ terminus, leading to the proposal that the function of Est1p is to mediate association of the 3′ terminus of the telomere with the Est2p active site. This may be essential for telomerase function in vivo, as null mutants of *EST1* and *EST2* have identical phenotypes (Lendvay et al. 1996); however, a test of this proposal will rely on the identification and phenotypic characterization of mutations of *EST1* that are specifically defective for telomeric DNA binding. Est1p also exhibits a nonspecific RNA binding activity in vitro, with no enhanced binding to the *TLC1* telomerase RNA (Virta-Pearlman et al. 1996), although this lack of specificity could be the result of incorrect folding of the 1.3-kb yeast telomerase RNA or the lack of another protein binding partner. None of these data currently distinguish between Est1p performing its function as a subunit of the enzyme or alternatively as a component of telomeric chromatin required to load telomerase onto the chromosomal terminus. For example, a bona fide interaction between Est1p and the telomerase RNA could still be a consequence of a contact between telomerase and telomere-bound Est1p, as part of a telomerase-loading activity.

A comparison of the in vitro properties of the yeast Est1 protein with those of the *Tetrahymena* p95 protein also invoke potential parallels. Despite a lack of primary sequence similarity, both proteins exhibit sequence-specific, low affinity, binding to single-strand telomeric DNA substrates, as well as nonspecific interactions in vitro with RNA (Virta-Pearlman et al. 1996; Gandhi and Collins 1998). This resemblance could indicate that Est1p, as proposed for p95, provides a specific site on the telomerase holoenzyme for primer binding that is separate from the telomerase active site. This model, of course, raises the question of why extracts that completely lack the Est1 protein still retain telomerase activity. However, mutant extracts have currently only been examined with saturating primer concentrations, potentially masking a requirement for Est1p in optimal telomerase activity. Similarly, if Est1p manifests its role as a component of telomeric chromatin, the use of naked DNA primers in the current telomerase assay rather than chromatin-bound DNA substrates would also fail to assess such a function. Biochemical experiments are in progress to investigate these possibilities.

Information about the specific role of the Est3 protein in telomere replication is not yet available, although the Est3 protein exhibits *EST2*-dependent association with the telomerase RNA, arguing that Est3 interacts directly with the enzyme (T. Hughes and V. Lundblad, unpubl.). The 20-kD Est3 protein has no discernible motifs or homologs in the database, and shows no sequence similarity to any of the telomerase-associated proteins identified in the ciliate systems. Characterization of how the *EST3* gene synthesizes its protein product has revealed an unexpected result, in that the same programmed translational frameshifting mechanism used by yeast retrotransposons is also employed to produce the full length Est3 protein (Morris and Lundblad 1997). *EST3* is the first example in yeast of a gene required for cellular growth that uses such a process, and raises questions about a potential connection between retrotransposition and telomere maintenance. Such a link had already been provided previously from studies of *Drosophila*, which unlike most organisms, does not rely on telomerase to maintain its telomeres. Instead, telomere-specific retrotransposons are used to replenish the ends of *Drosophila* chromosomes (Biessmann et al. 1990; Levis et al. 1993), with one of these elements relying on ribosome frameshifting to maintain its protein product (Danilevskaya et al. 1992). Isolation of *EST3* genes in other organisms will be necessary to determine whether frameshifting is a conserved feature of this telomere replication protein.

Although characterization of such proteins in these three organisms has resulted in one clear-cut convergence (the catalytic subunit of the *Euplotes* and yeast enzymes), equally striking is the overall lack of homologs. Although recent evidence suggests that the *Tetrahymena* telomerase also has a RT catalytic subunit (T. Bryan and T. Cech; M. Rudd and C.W. Greider; both pers. comm.), it is unclear why the *Euplotes* enzyme is not associated with p95 and p80-like proteins, nor the *Tetrahymena* enzyme with a p43 subunit. Particularly curious is the fact there is no obvious p80 yeast homolog detectable by searching the completed sequence of the *S. cerevisiae* genome, even though the identification of human, rat and murine p80 homologs has eliminated the possibility that p80 is a ciliate-specific telomerase protein. This lack of convergence may simply be a technical consequence of how early we are in our understanding of what constitutes a telomerase holoenzyme in any system, with additional components still to be identified in each organism. Alternatively, these differences may be attributable to biological variation; despite the overall conservation of a RT with an intrinsic RNA subunit, telomerases in different organisms clearly need to respond to differing requirements, as exemplified by the substantial species-specific variations in telomere length. For example, *Euplotes* telomere length is tightly regulated (28 bp of duplex with a 14-nucleotide G-rich overhang; Klobutcher et al. 1981), whereas telomere length in *Tetrahymena* and yeast is both longer and less stringently controlled, ranging from ~300 to 500 bp (Larson et al. 1987; Shampay and Blackburn 1988). Even the structure of the p80 homolog is not precisely conserved, as the mammalian versions are substantially larger (230–240 kD), suggesting additional as yet undetermined function(s).

The consequences of the absence of telomerase

An early prediction for the phenotype of telomerase defective cells was that a telomerase deficiency should not be immediately lethal. This was based on the assump-

tion that loss of a substantial amount of the duplex telomeric G-rich tract could occur before telomere function would be compromised, a premise that was first experimentally verified in the unicellular yeasts. As was initially shown for the *S. cerevisiae est1*$^-$ mutant, *S. cerevisiae*, *K. lactis*, and *S. pombe* strains defective for the telomerase pathway are initially viable, but the resulting telomere shortening leads to eventual cellular senescence in each of these experimental organisms (Lundblad and Szostak 1989; Singer and Gottschling 1994; McEachern and Blackburn 1995; Lendvay et al. 1996; Nakamura et al. 1997). One implication of this delayed cell death phenotype is that the structure found at the very terminus of the telomere (and hence the presumed telomere capping function) continues to be maintained in the absence of telomerase (Wellinger et al. 1996); proteins that maintain the protective telomeric cap are considered in the last section of this review.

These studies also demonstrated that a defect in telomere replication was sufficient to impose a finite life span on the normally immortal growth characteristics of wild-type yeast. Similarly, although some human cell types with indeterminate proliferative capacity, such as germ-line cells, express telomerase, not all cells sustain an active mechanism to maintain stable telomere length. Telomerase is not detectable in most human somatic cells and presumably as a consequence, telomere length recedes as cells replicate (Cooke and Smith 1986; Harley et al. 1990; Hastie et al. 1990; Klm et al. 1994). This led to the proposal that the inability to maintain telomere length and/or the terminal structure eventually limits the proliferation of these cells, both in vivo and in cell culture (Olovnikov 1973; Cooke and Smith 1986; Harley 1991). The cloning of the human reverse transcriptase subunit and the demonstration that this component is limiting in normal human cells has allowed a definitive test of this hypothesis, by asking whether stable expression of the hTERT protein extends cell life span in culture. Conversion of two different primary cell types to telomerase-plus results in telomere elongation; more strikingly, these clones fail to exhibit the standard characteristics of senescence after additional propagation (Bodnar et al. 1998). Instead, clones expressing hTERT bypassed the expected senescence point, exceeding their normal life span by at least 20 population doublings. In addition, these extended life span clones have so far maintained a normal karyotype and display the phenotypes associated with young cells. This pivotal result suggests a molecular basis for the long-ago observed Hayflick limit that dictates replicative senescence in culture (Hayflick and Moorhead 1961) by showing that restoration of telomerase activity can influence the mitotic clock that determines life span. This further supports the concept that reactivation of telomerase may be a primary means to promote immortality during oncogenesis, by removing the short telomere barrier to tumor progression.

An alternative mammalian model that has allowed an examination of the in vivo consequences of a telomerase deficiency has stemmed from the creation of knockout mice lacking the telomerase RNA subunit (Blasco et al. 1997). The initial striking observations from this experiment are that these mice are not only viable but also fertile for up to six generations, with progeny displaying at least modest telomere shortening as well as an increased incidence of chromosome abnormalities in late generations. Analysis of this mutant strain, however, has been complicated by the fact that the laboratory strain of wild-type *Mus musculus* mice has extremely long telomeres, reaching lengths ≤40 kb (Prowse and Greider 1995). This has necessitated extensive homozygous back-crossing to permit telomere shortening to a length more comparable to that of human cells, with the expectation that phenotypes not evident initially would now become apparent. This approach has in fact revealed that cells that normally display high proliferation rates in the wild-type mouse are now at a disadvantage in the late backcrosses (Lee et al. 1998). This argues that telomere length maintenance plays a role in long-term cellular proliferation not only in cell culture but also in vivo in multicellular organisms.

Expression of telomerase, however, is not the only route to maintaining the ends of linear chromosomes. Experimental studies have shown that in the absence of telomerase, yeast has the ability to mobilize an alternative mechanism for restoring G-rich sequences to the telomere. Although the majority of *est*$^-$ mutant cells die during extended propagation of the culture, a small subset escape the lethal consequences of a telomerase deficiency. These survivors arise as a result of recombination-mediated global amplification of telomeric G-rich repeats as well as adjacent subtelomeric regions (Lundblad and Blackburn 1993; Lendvay et al. 1996). The effect of this genomic reorganization can be quite dramatic, resulting in up to a 40-fold increase in telomeric G-rich repeats in some cases (as well as an increase in subtelomeric repeats), such that 4% of the genome consists of telomeric DNA in certain *est*$^-$ survivors. Successive rounds of recombination between these expanded telomeres has been proposed as a means of continually replenishing the telomere, whereby telomere structure is now maintained by recombination rather than by telomerase (Lundblad and Blackburn 1993). This process is not specific to telomere replication defects in *S. cerevisiae*, as a similar telomerase bypass pathway has been observed in *K. lactis* (McEachern and Blackburn 1996), and potential parallels may occur in mammalian cells as well. Both a substantial number of established human cell lines, as well as in vivo tumors, are telomerase-minus, but despite the lack of enzyme activity, telomeres are still exceptionally long (Kim et al. 1994; Bryan et al. 1995, 1997). Although the basis for this alternative mechanism for telomere lengthening (dubbed ALT) has not been elucidated, it further demonstrates that there are nontelomerase options available for chromosome end maintenance. It is also possible that the very long telomeres of *M. musculus* mice arose at least partly through nontelomerase mechanisms; the telomerase RNA knockout mouse may provide opportunities to examine the contribution of both telomerase and non-

telomerase based processes to mammalian telomere maintenance.

The above observations come from studies of organisms that normally utilize telomerase, but there is also a naturally occurring example of alternative telomere maintenance. *Drosophila*, used in Muller's pioneering studies to define the telomere, does not employ telomerase and there is no evidence for the G-rich telomeric repeats found in most organisms (Table 1) at the ends of *Drosophila* chromosomes. Instead, the sequence of *Drosophila* telomeres are a complex pattern of retrotransposable elements, with telomere maintenance apparently due to a balance between gradual loss of DNA and reinsertion of telomere-specific non-LTR retrotransposons (Biessmann et al. 1990; Levis et al. 1993). It is tempting to speculate that this process of telomere maintenance is related to telomerase. Both processes elongate chromosomal termini by the addition of RNA-templated DNA, and as mentioned above, the RTs of non-LTR retrotransposons and the telomerase reverse transcriptase appear to be both mechanistically and evolutionarily related (Nakamura et al. 1997; Eickbush 1997). If there is a direct relationship, phylogenetic arguments do not currently differentiate between whether a retrotransposon-based mechanism replaced *Drosophila* telomerase, or vice versa. As the details of both processes become available, this may address the potential evolutionary relationship.

Interactions between telomerase and proteins that protect the telomere

A primary function of the telomere, as well as at least a subset of associated proteins, is to protect chromosomal termini from end-to-end fusion and severe telomere erosion. That chromosomes in cells lacking telomerase activity do not immediately undergo gross rearrangements or exhibit instability attests to the presence of a telomerase-independent mechanism operating to prevent such events. Therefore, in parallel with the characterization of telomerase, identification of the protein(s) responsible for providing the protective cap function of telomeres has been an area of intense investigation. These capping proteins also have the potential to interact, either directly or indirectly, with telomerase and regulate its activity. Conversely, because telomerase is responsible for generating the G-rich telomeric repeat tract to which telomeric proteins bind, this suggests the possibility of a dynamic and highly complex set of regulatory interactions.

In a wide variety of organisms, a specific structure has been observed at chromosomal termini, with the G-rich strand protruding as a single strand extension (Wellinger and Sen 1997). In several different species, this structure has been shown to be complexed with proteins, one or more of which could conceivably contribute to the hypothesized protective telomeric cap. The first single-strand end binding proteins to be characterized in detail have, like telomerase, been recovered from the ciliates. These activities, best studied in *Oxytricha* and *Euplotes*, bind tenaciously to single-strand telomeric repeats and are terminus-specific in vivo and in vitro (Fang and Cech 1995). Physical evidence supporting the hypothesis that these proteins form a protective structure is the resistance of bound telomeric DNA to nuclease digestion and chemical modification (Gottschling and Zakian 1986; Price and Cech 1987). Terminal proteins such as these also have the potential to interact or compete with telomerase, regulating its activity, but the limitations of ciliate genetics have prevented an in vivo test of either proposed role.

In yeast, both in vivo and in vitro data suggest a function at the telomeric terminus for two single-strand telomeric DNA binding proteins, Est1 and Cdc13. However, although Est1 fulfills the biochemical criteria for a terminus-specific telomere binding protein, its mutant phenotype suggests that it has no more of a role in chromosome end protection than does *EST2* (Virta-Pearlman et al. 1996; Lendvay et al. 1996). A far better candidate for a yeast end-binding activity is the Cdc13 protein. First identified in Hartwell's classic cell division cycle collection of mutants (Hartwell and Smith 1985), the absence of *CDC13* function results in catastrophic and immediate loss of sequences from the C-strand of the telomere (Garvik et al. 1995). Consistent with a role in maintaining telomere integrity, Cdc13p binds single-strand telomeric DNA in vitro (Lin and Zakian 1996; Nugent et al. 1996). The function of *CDC13* goes beyond telomere protection: The characterization of additional alleles of *CDC13* argues for a complex regulatory interaction with telomerase as well. The *cdc13-2*^{est} allele, isolated in the screen that identified the *EST* genes, exhibits the same in vivo telomerase deficiency as other *est* mutants (Lendvay et al. 1996; Nugent et al. 1996), although enzyme activity is still present in vitro (Lingner et al. 1997b). This has led to the model that Cdc13 plays a dual role at the telomere: It not only provides end-binding protection but has a separate role in positively regulating access of telomerase to the chromosomal terminus (Nugent et al. 1996). The phenotypes of yet a third type of mutation in *CDC13*, which results in greatly elongated telomeres, argue that *CDC13* also mediates not only positive regulation but also negative regulation of telomere length (T. Hughes and V. Lundblad, unpubl.). This initial analysis of *CDC13* has already provided a picture of a protein that, in its proposed position at the chromosome terminus, participates in an intricate set of interactions involving both telomere length maintenance and telomere protection.

Although proteins that bind to the very terminus are the most logical candidates for providing the "cap", recent work from the de Lange laboratory has provided striking evidence that proteins bound to the duplex portion of the telomere play a pivotal role in protecting chromosome ends from fusion (van Steensel et al. 1998). Two TTAGGG repeat binding factors, TRF1 and TRF2, have been characterized previously in human cells (Chong et al. 1995; Bilaud et al. 1997; Broccoli et al. 1997). TRF1, a homodimeric protein with a Myb-like DNA binding domain, is a negative regulator of telomere

length and has been proposed to control telomere elongation via *cis*-mediated inhibition of telomerase (van Steensel and de Lange 1997). TRF2 has a similar Myb motif and also binds human duplex telomeric repeats in vitro, but is distinguished from TRF1 by its amino-terminal domain (Bilaud et al. 1997; Broccoli et al. 1997). Overexpression of dominant-negative alleles of TRF2 in human cells results in loss of terminal single-strand 3′ overhangs (although the duplex stretch of TTAGGG repeats is retained), with an accompanying sharp increase in the frequency of end-to-end chromosome fusions (van Steensel et al. 1998). In addition to protection against chromosome end fusions, TRF2 also affects the proliferative potential of cells, as expression of mutant TRF2 proteins leads to irreversible growth arrest of human fibrosarcoma cells, resulting in a senescence-like phenotype (van Steensel et al. 1998). These observations indicate that TRF2 is a key mediator of the loss of telomere function and growth arrest that result from telomere shortening due to the absence of telomerase. This also raises the intriguing question of whether duplex telomere binding proteins in other organisms may play similar roles.

Perspectives

The past several years have seen substantial advances in our understanding of how organisms with linear chromosomes replicate their ends. Central to this progress has been the discovery of the catalytic subunit of telomerase. A remarkable consequence of this finding has been the realization that a reverse transcriptase plays an essential role in chromosomal replication and cellular growth. Equally important, this now provides the foundation for a complete understanding of the telomerase complex. In parallel, the characterization of a number of telomerase-associated proteins promises to extend studies of the telomerase holoenzyme. The identification of these proteins will allow the field to tackle the next challenges: unraveling the precise mechanism of telomerase catalysis, understanding the means by which telomerase activity is regulated, and determining the relevant factors that allow telomeres to contribute to chromosome transmission.

However, although the excitement of the last few years has focused heavily on telomerase, the novel mechanism of telomere maintenance in *Drosophila* and the ready appearance of telomerase bypass pathways indicates that a cell can find alternative ways to maintain its termini, when faced with the selective pressure of replicating its genome. This certainly raises the question of how many other telomere maintenance mechanisms are used by less widely studied research organisms. Just as studies in *Tetrahymena* were judged (erroneously) early on to be novelty items, delving into the terminal structures of organisms that deviate from the telomerase norm may be where the new discoveries await.

In human cells, the data suggest that attrition of telomeres may impose a limit upon cell proliferation, presenting a barrier to tumor cell growth. Although the molecular details of how telomere length might signal a tumor suppressor system are still unclear, the TRF2 protein appears to play a pivotal role in this process. It seems reasonable to propose that mammalian end binding protein(s) similar to the yeast Cdc13 protein will also be involved, as well as other telomere-localized proteins yet to be identified. Future detailed understanding of the factors that regulate this process will be critical not only for telomere biologists but also for those who want to apply such findings to research on cancer and aging.

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